

Impact of Bt Cottons Expressing One or Two Insecticidal Proteins of *Bacillus thuringiensis* Berliner on Growth and Survival of Noctuid (Lepidoptera) Larvae

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ABSTRACT A series of laboratory assays were performed to compare the relative impact of commercial and experimental cultivars of cotton, *Gossypium hirsutum* (L.), expressing zero, one, or two insecticidal proteins of *Bacillus thuringiensis* Berliner, on several lepidopteran pests. Assays in which larvae were fed fresh plant tissue indicated that dual-toxin *B. thuringiensis* (Bt) cultivars, expressing both Cry1Ac and Cry2Ab endotoxins of *B. thuringiensis*, were more toxic to bollworms, *Helicoverpa zea* (Boddie), fall armyworms, *Spodoptera frugiperda* (J. E. Smith), and beet armyworms, *Spodoptera exigua* (Hübner), than single-toxin cultivars expressing Cry1Ac. Assays in which lyophilized plant tissue was incorporated into artificial diet also indicated improved activity of the dual-toxin Bt cultivar compared with single-toxin plants. Both bollworm and tobacco budworm, *Heliothis virescens* (F.), growth was reduced by Bt cotton, particularly the dual-toxin cultivar. Although assays with lyophilized tissues were done using largely sublethal doses, bollworm survival was reduced by the dual-toxin cultivar. It appears that this newly developed Bt cotton expressing two toxins will be more effective and have a wider range of activity on these lepidopteran pests.

KEY WORDS transgenic *Bacillus thuringiensis* cotton, Cry1Ac, Cry2Ab, Lepidoptera, toxicity

TRANSGENIC BT COTTON, *Gossypium hirsutum* (L.), expressing the Cry1Ac insecticidal δ -endotoxin of *Bacillus thuringiensis* Berliner, has been commercially available in the United States since 1996. Since then, *B. thuringiensis* (Bt) cotton has demonstrated remarkable control of some lepidopteran pests, particularly the tobacco budworm, *Heliothis virescens* (F.), and the pink bollworm, *Pectinophora gossypiella* (Saunders). Since its release into commercial markets, Bt cotton seldom, if ever, has required supplemental insecticide control for these two pests (Williams 2000). Control of the bollworm, *Helicoverpa zea* (Boddie), has been less dependable, and economically damaging infestations of this pest can occur on Bt cotton (Mahaffey et al. 1995), particularly after plants have begun flowering and when insecticides have disrupted populations of predators and parasites (Lambert et al. 1996, Turnipseed and Sullivan 1999). The bollworm is inherently more tolerant than tobacco budworm to the Cry1Ac δ -endotoxin expressed in currently available Bt cultivars (Luttrell et al. 1999). Additionally, low expression of the toxin in some plant parts, such as in pollen and petals, has been implicated in increased survival of the bollworms on Bt cotton (Adamczyk et al. 2000). Other common lepidopteran pests [e.g., fall armyworms, *Spodoptera frugiperda* (J. E. Smith); beet armyworms, *Spodoptera exigua* (Hübner); and soybean loopers,

Pseudoplusia includens (Walker)] are even more tolerant than bollworms to Cry1Ac (Luttrell et al. 1998, 1999).

One advantage of Bt cotton is that it greatly reduces injury and insecticide use associated with control of tobacco budworms and bollworms (Stewart et al. 1998). However, in low-spray environments, pests like soybean loopers, beet armyworms, and fall armyworms can potentially thrive, even in Bt cotton (Adamczyk et al. 1998, Ashfaq and Young 1999, Stewart et al. 2000). The insertion of an additional Bt gene encoding for a different toxin (Cry2Ab; Monsanto, St. Louis, MO) into current Bt cotton cultivars containing Cry1Ac may provide additional activity on certain lepidopteran pests and would improve the pest-control value of Bt cotton.

The potential impact on resistance management of cotton that expresses multiple toxins has been addressed (Tabashnik 1994, Sachs et al. 1996, Gould 1998). Mandating a refuge of non-Bt cotton as a source of susceptible individuals to mate with resistant insects has been the primary approach for preventing resistance to Bt cotton (Caprio 1994). This strategy relies on Bt cotton expressing a "high dose" (e.g., > 25 fold the LD₉₉) to lepidopteran pests so that survivors in Bt cotton are rare and heterozygous individuals are rendered functionally recessive (Gould 1998). This tactic has been criticized because commercially available Bt cottons do not appear to meet the high-dose criterion for bollworm as it presumably does for tobacco bud-

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worm and pink bollworm. A potential advantage of having two Bt toxins expressed in cotton is that the risk of insect pests developing resistance to Bt cotton may be reduced, especially if resistance to one toxin does not confer resistance to the other.

Although not yet commercially available, transgenic cotton lines expressing two Bt toxins are under development. This article reports the results of laboratory assays with a dual-toxin Bt cotton (Cry1Ac and Cry2Ab) compared with single-toxin Bt cottons (Cry1Ac) and non-Bt cottons. Our primary objective was to compare the relative toxicity of single- and dual-toxin Bt cotton cultivars on tobacco budworm, fall and beet armyworm, and particularly bollworm. A secondary objective was to determine assay methods that are best suited for quantifying differences in the relative toxicity of Bt cottons.

Materials and Methods

Source of Plant Materials and Larvae Used in Assays. DPL50 (non-Bt), DPL50B (expressing Cry1Ac), and MON15985 and MON15813 (expressing Cry1Ac and Cry2Ab) were the primary, near-isogenic cultivars used in our assays. Other cultivars were used in some comparisons as indicated below. All cottons were Delta and Pine Land (Scott, MS) cultivars provided to the researchers in 1999 by the Monsanto. All plant materials used in fresh tissue assays were collected from field plots during 1999.

Fresh tissue assays with neonate and third-instar bollworms were done at the USDA-ARS-SIMRU (Southern Insect Management Research Unit, Stoneville, MS). All bollworms used in testing were from a colony created in the spring of 1999 when ≈ 300 individuals were collected from field corn near Stoneville, MS. The laboratory rearing of bollworms was conducted as described in Adamczyk et al. (1998), and the G_1 or G_2 generation was used in assays. Replicated field plots (Shelby, MS) containing DPL50, DPL50B and NuCOTN 33B (expressing Cry1Ac), and MON15985 and MON15813 (expressing Cry1Ac and Cry2Ab) were the source of plant tissue used in these experiments. In addition, replicated field plots (Elizabeth, MS) containing eight non-Bt and 17 Bt cotton cultivars were also used to make comparisons with dual-toxin Bt cotton. Field plots were maintained by standard agronomic practices for the region, including herbicide and nitrogen applications.

Assays using second-instar bollworm, beet armyworm, and fall armyworm were done at Mississippi State University (Mississippi State, MS). Additional experiments with neonate bollworm and tobacco budworm, using lyophilized leaf tissue, were also done at Mississippi State University. Larvae used were from the USDA-ARS-CHPRRU (Corn Host Plant Resistance Research Unit, Mississippi State, MS). These are long established colonies maintained as described by Davis (1989). Unless indicated, replicated field plots of DPL50, DPL50B, and MON15985 at Mississippi State University were used as a source of plant tissue in

these assays. Field plots were established and maintained as described by Stewart et al. (2000).

When field plots were used as a source of plant material for assays, plant tissues were collected equally from insecticide-untreated replicates. Throughout all experiments, larvae were maintained at $28 \pm 2^\circ\text{C}$, 70–80% RH, and a photoperiod of 14:10 (L:D) h.

Fresh Tissue Assays (Neonate Bollworm). To examine if dual-toxin Bt cotton (MON15813 and MON15985) was more toxic than single-toxin Bt cotton (17 different commercial cultivars expressing Cry1Ac) to neonate bollworms, leaves from plants in the fourth true leaf stages were harvested and fed to larvae in the laboratory. In addition, we used non-Bt commercial cultivars (8) as controls. Fully expanded leaves (15) were harvested from each replicate (four replicates per cultivar) with ≈ 0.5 cm of petiole remaining to prevent desiccation and transported to the laboratory in a cooler containing ice. Within 2 h of harvesting, a single leaf was placed into individual rearing cups (30 ml, JetPlastica, Hattisburg, PA), and a neonate bollworm was placed into each cup, which was then capped (60 neonates/cultivar). All cups were placed inside an autoclave bag (model 12 by 24 inches (30.5 by 61.0 cm), clear polyethylene; Fisher; Norcross, GA) to reduce leaf desiccation. Two days after exposure to cotton leaves, survival was recorded (i.e., movement present after disturbed with a camel's-hair brush). Fresh leaves, collected as before, were provided to larvae and this time. Survival was reassessed after two additional days.

Cry1Ac expression in flower parts is low compared with other plant parts (Adamczyk et al. 2000), and bollworms are commonly associated with flowers (J.J.A., unpublished data). Thus, a study was designed to examine if flower parts from the dual-toxin Bt cultivars were more toxic to neonate bollworms than flowers from single-toxin Bt cultivars. During the second week of flowering (peak flowering period), first position pink flowers were harvested from plots in Shelby, MS (three replicates), of NuCOTN 33B (expressing Cry1Ac) and MON15985 (expressing Cry1Ac and Cry2Ab) and transported to the laboratory within a cooler containing ice. To help make finding larvae easier, the petals were removed from the flowers. Within 2 h after harvesting, a single petal-less pink flower was placed into individual cups (30 ml), and two neonate bollworms were placed into each cup (60 flowers per cultivar, 120 neonates/cultivar). Survival was recorded at 2 d after larvae were infested onto the plant parts, and flowers were changed in cups containing live larvae, using flowers collected before. Survival was reassessed after two additional days. For the leaf and pink flower assays, mean survivorship was analyzed using analysis of variance (PROC REML-ANOVA, SAS Institute 1998), and means were separated via the lsmeans option of PROC Mixed (Littell et al. 1996). Numbers of live larvae were log transformed before analysis because treatment variances were not homogeneous.

Because it is widely believed that larvae move from flowers to bolls once flowers begin to desiccate, a study was designed to examine if increased efficacy on bollworm neonates was observed with dual-toxin Bt cotton on 1-d-old bolls. First position white flowers (30) from DPL50, NuCOTN 33B, and MON15985 plants were randomly tagged (Slant 'N Lock; A.M. Leonard, Piqua, OH [as described in Adamczyk et al. 1998]) in field plots at Shelby, MS. After 24 h, the 1-d-old bolls were removed from the plant and transported to the laboratory within a cooler containing ice. All remaining flower parts, including bracts and stems, were removed from the bolls, and the bolls were washed with distilled water and allowed to air dry. The bolls were then placed into individual cups (30 ml), and a single neonate was introduced. Survival of neonates was rated 4 d after they were infested onto plant parts and analyzed using PROC Freq with likelihood ratio chi-square (SAS Institute 1998). Survival differences among cultivars were separated using Fisher exact test (two-tailed) (Steel and Torrie 1980).

Fresh Tissue Assays (Second-Instar Bollworm, Beet Armyworm, and Fall Armyworm). Two similar methods were used to evaluate the relative toxicity of DPL50, DPL50B, and MON15985 on bollworm, fall armyworm, and beet armyworm. The two assay methods differed only in that in one method the insects were allowed to feed on plant tissue for 48 h and were then transferred to artificial diet (King and Hartley 1985), and in the other method, new plant tissue was provided to larvae every 48 h. Plant parts were collected from field plots, returned to the laboratory, washed with tap water, and air-dried before use in assays.

Second-instar larvae previously maintained on artificial diet (King and Hartley 1985) for 48 h post-hatch were fed various plant parts. Bollworms and fall armyworms were given freshly picked white flowers because they primarily feed on fruiting structures. Beet armyworms were fed fully expanded leaves from the four to sixth node below the terminal because they are primarily leaf-feeders in cotton. Our assay method was similar to that of Chang et al. (2000). Approximately 0.5 cm of 2.0% sterile agar was poured into petri dishes (9 cm diameter), and autoclaved paper toweling was placed over the agar. This paper was cut to fit the bottom of the dishes and served as a wick for the moisture in the agar. The plant material was trimmed so that it would fit into the dishes and maintain contact with the toweling. The plant parts and one larva were placed in each dish, with the larvae placed on top of the plant tissue. Dishes were covered with corresponding lids. For each of the three species and cultivars, 60 larvae were tested. A few larvae escaped or were lost during the study, and these were omitted from analyses.

After 48 h, survival and the relative amounts of feeding damage (0–5 scale, no damage to total consumption) to the plant parts were recorded for each individual. Approximately one-half of the surviving insects were transferred to 30-ml plastic rearing cups (JetPlastica Co., Hattisburg, PA) containing ≈ 5.0 ml

artificial diet. The remaining larvae were provided additional plant material at 48-h intervals until death or pupation. Survival was again determined 24 h later and subsequently at 48-h intervals until pupation. The lengths of larvae (mm) were recorded 5 and 7 d after their initial placement on plant tissue. Dates of pupation were also recorded for all surviving insects.

Our primary interest was to determine cultivar effects on the survival and growth of common lepidopteran pests such as bollworm, beet armyworm, and fall armyworm. Statistical comparison about how different plant parts influenced insect survival and development were not done because these experiments were not always done at the same time or with the same cohort of insects. Also, no attempt was made to statistically compare across species because different plant parts were used for testing. Besides cultivar effects, we also were interested in comparing the two assay methods (i.e., larvae transferred to artificial diet and those continually fed plant material). Thus, cultivar and assay methods were the two factors included in analysis. Each larva represented a replicate. Data for survival and the relative amount of feeding on plant tissue were categorical; therefore, we used categorical data analysis and protected lsmeans for separation of treatment effects (PROC Genmod, SAS Institute 1998). Cultivar effects on larval length and time to pupation were analyzed with ANOVA and using Student-Newman-Keuls for mean separations (PROC GLM, SAS Institute 1998).

Fresh Tissue Assays (Third-Instar Bollworm). Because bollworms may have the ability to survive on Bt cotton plant parts expressing low levels of Bt (i.e., flowers) or immigrate from non-Bt cotton, an experiment was designed to test the effect dual-toxin Bt cotton had on older bollworm larvae when fed 4-d-old bolls. First position white flowers were tagged in field plots at Shelby, MS, as previously described. After 4 d, the corresponding bolls (15, 25, and 35 for DPL50, NuCOTN 33B, and MON15985, respectively) were harvested. Bioassays were done as described for pink flowers except larvae were reared initially to the third instar on artificial diet (King and Hartley 1985), and only one larva was placed in each cup. Survival of neonates as well as number of bolls penetrated (i.e., penetration of boll wall to seed/fiber) were rated at 2, 4, and 6 d after infestation on plant tissue and analyzed using PROC Freq with likelihood ratio chi-square (SAS Institute 1998). Differences in survival among cultivars were separated using Fisher's exact test (two-tailed) (Steel and Torrie 1980).

Assays with Lyophilized Leaf Tissue (Neonate Bollworm and Tobacco Budworm). Bioassays using lyophilized leaf tissue that was incorporated into artificial diet were done with neonate tobacco budworms and bollworms to compare the sensitivity of these species to DPL50, DPL50B, and MON15985. In the first repetition of this experiment, done in 1999, 100 leaves located four to five nodes below the terminals were collected from the field plots at Mississippi State University. These leaves were collected when plants were near physiologic cutout (Harris et al. 1997). At

Table 1. Proportion of neonate bollworms surviving 2 and 4 d when fed cotton containing zero, one (Cry1Ac), or two Bt proteins (Cry1Ac and Cry2Ab)

Plant part	Cultivars	Survival		n
		2 d	4 d	
Leaves ^a	Non-Bt ^b	0.96 ± 0.01a	0.92 ± 0.01a	480
	Bt ^c	0.60 ± 0.03b	0.34 ± 0.03b	1,020
	BtII ^d	0.38 ± 0.07c	0.07 ± 0.02c	120
Pink flowers ^e	NuCOTN 33B ^f	0.72 ± 0.05a	0.48 ± 0.04a	120
	MON15985 ^e	0.55 ± 0.05a	0.18 ± 0.03b	120
1-d-old bolls ^h	DPL50	—	0.83a	30
	NuCOTN 33B ^f	—	0.10b	30
	MON15985 ^e	—	0.00b	30

Analyses for leaves and pink flowers were done on number of surviving insects per replicate following log transformation (PROC Mixed, Littell et al. 1996). Means ± SE within columns and plant parts not followed by a common letter are significantly different (lsmeans, $P < 0.01$). Data for 1-d-old bolls were analyzed with PROC Freq (SAS Institute 1998). Numbers not followed by a common letter are significantly different [Fisher's exact test (two-tailed), $P < 0.05$, Steel and Torrie 1980]. n, Number of insects tested.

^a ANOVA results: 2d- $F = 44.4$; df = 2, 102; $P < 0.01$. 4 d- $F = 86.8$; df = 2, 102; $P < 0.01$.

^b Mean from eight commercial non-Bt cultivars, Elizabeth, MS.

^c Mean from 17 commercial Bt cultivars (Cry1Ac), Elizabeth, MS.

^d Mean from two experimental Bt cultivars, MON15813 and MON15985 (Cry1Ac and Cry2Ab), Shelby, MS.

^e ANOVA results: 2d- $F = 2.3$; df = 1, 4; $P = 0.21$. 4d- $F = 2.3$; df = 1, 4; $P < 0.01$.

^f Commercial Bt cultivar (Cry1Ac).

^g Experimental Bt cultivar (Cry1Ac and Cry2Ab).

^h Likelihood ratio chi-square (PROC Freq, SAS Institute 1998). $\chi^2 = 65.1$, df = 2, $P < 0.01$.

physiological cutout, plants have a near maximum fruit load, resulting in slowed vegetative growth. Greenhouse-raised plants were used during the second repetition of the experiment, which was done in April 2000. Greenhouse plants, two per pot, were watered twice weekly and kept at 20–30°C without supplemental lighting. Plants were fertilized once (Osmocote 14–14–14, N:P:K, Scotts, Columbus, OH). Fifty fully expanded leaves per cultivar were collected from ≈50 preflowering plants. All leaves were returned to the laboratory, freeze dried, and stored at –80°C until use.

Lyophilized leaves were ground into a powder using a mortar and pestle. For each of the three cultivars, the tissue was incorporated into artificial diet using a high-speed blender. Sublethal doses (1.124, 11.24, 112.4, and 1124 µg plant tissue/ml diet) were used to compensate for the high susceptibility of these insects, particularly tobacco budworm, to Bt cotton. For each dose, and for both repetitions of the experiment, 96 tobacco budworm and bollworm neonates were individually placed in 5.0-ml wells containing ≈2 ml of treated diet. Trays containing 24 wells were covered with shrink wrap plastic and heat sealed. Survival of larvae was determined 3 and 7 d after infestation on diet. Larval length (mm) was measured at 7 d for six randomly selected larvae from each tray. Survival data were analyzed using PROC Genmod and lsmeans for separation of treatment effects (SAS Institute 1998). ANOVA was used to determine treatment effects on larval length, and Student–Newman–Keuls was used for mean separations (PROC GLM, SAS Institute 1998).

Results

Fresh Tissue Assays (Neonate Bollworm). Neonate bollworms were more susceptible to leaves and pink

flowers from dual-toxin Bt cultivars (Cry1Ac and Cry2Ab) than those from non-Bt or single-toxin Bt cultivars (Cry1Ac), when mortality was assessed after 4 d (Table 1). After 2 and 4 d, survival of neonates fed leaves of dual-toxin Bt cultivars was less than those fed single-toxin Bt or non-Bt cultivars (Table 1). Higher survival rates were also found for larvae fed leaves of non-Bt cotton compared with single-toxin Bt cultivars. Although there was not a significant difference after 2 d, more larvae were alive after 4 d when fed pink flowers of a single-toxin Bt cultivar (NuCOTN 33B) compared with a dual-toxin Bt cultivar (MON15985).

A higher survival rate (Table 1) was observed for neonates fed 1-d-old bolls from non-Bt cotton (DPL50) compared with either Bt cultivar (NuCOTN 33B and MON15985). However, no significant differences were observed between the single and dual-toxin Bt cultivar.

Fresh Tissue Assays (Second Instar Bollworm, Beet Armyworm, and Fall Armyworm). Our results indicate that dual-toxin Bt cotton, compared with the non-Bt and the single-toxin cultivars, reduced the survival of second-instar larvae. Across all species and assays, cultivar effects on survival were highly significant ($\chi^2 = 60.7$, df = 2, $P < 0.01$). The fewest larvae (36%) survived to the pupal stage when fed MON15985 than when fed plant material from DPL50B (64%) or DPL50 (77%). DPL50B had less of an impact on survival than MON15985 ($\chi^2 = 26.7$, df = 1, $P < 0.01$). The greater effect of the dual-toxin cultivar on survival was especially apparent for beet armyworms (Table 2). Only 39% of the beet armyworms fed tissue from the MON15985 cultivar survived to pupation, but 93% of larvae survived when fed tissue from DPL50B. At 3, 7, and 15 d, the survival of both bollworm and fall armyworm was statistically less when fed MON15985 than when fed DPL50B. However, it should be noted that fall armyworm survival on

Table 2. Relative feeding, survival, mean larval lengths (mm \pm SE), and delays in pupation when second-instar bollworm, fall armyworm, and beet armyworm were fed plant parts from cotton containing zero (DPL50), one (DPL50B, Cry1Ac), or two Bt proteins (MON15985, Cry1Ac and Cry2Ab)

Species	Plant part	Cultivar	n	Relative feeding ^a	Proportion of larvae surviving				Larval length (mm) after:			Pupation delay (d) ^b
					3 d	7 d	15 d	To pupa	5 d	7 d		
Bollworm	Flowers ^c	DPL50	59	1.72 \pm 0.17 ^a	0.85 ^a	0.82 ^a	0.67 ^a	0.65 ^a	15.6 \pm 0.5 ^{a,m}	24.1 \pm 0.6 ^{a,m}	0.00 \pm 5.95 ^a	
		DPL50B	58	0.47 \pm 0.07 ^b	0.26 ^b	0.19 ^b	0.16 ^b	0.07 ^b	6.3 \pm 1.0 ^{b,m}	16.5 \pm 1.7 ^{b,m}	8.60 \pm 1.19 ^b	
Fall armyworm	Flowers ^c	MON15985	58	0.40 \pm 0.07 ^b	0.10 ^c	0.07 ^{c,m}	0.00 ^b	0.00 ^b	7.0 \pm 1.3 ^b	17.8 \pm 1.9 ^b	—	
		DPL50	60	2.25 \pm 0.08 ^a	1.00 ^a	0.90 ^{a,b}	0.86 ^{b,c}	0.75 ^b	19.3 \pm 0.5 ^{a,i}	21.0 \pm 0.7 ^{a,i}	0.00 \pm 0.30 ^{a,i}	
Beet armyworm	Flowers ^c	DPL50B	60	1.82 \pm 0.07 ^b	1.00 ^a	0.95 ^a	0.95 ^a	0.90 ^a	18.8 \pm 0.5 ^{a,m}	19.8 \pm 0.7 ^a	-0.67 \pm 0.24 ^a	
		MON15985	60	1.35 \pm 0.07 ^c	0.90 ^b	0.86 ^b	0.83 ^c	0.69 ^b	14.2 \pm 0.4 ^{b,m}	17.0 \pm 0.8 ^b	2.16 \pm 0.55 ^{b,m}	
	Leaves ^d	DPL50	57	1.25 \pm 0.07 ^a	1.00 ^a	1.00 ^a	0.89 ^a	0.88 ^{a,i}	19.1 \pm 0.4 ^{a,m,i}	21.1 \pm 0.4 ^{a,i}	0.00 \pm 0.16 ^{a,i}	
		DPL50B	57	1.02 \pm 0.05 ^b	0.98 ^a	0.96 ^a	0.93 ^a	0.93 ^a	17.7 \pm 0.7 ^{b,m}	19.7 \pm 0.7 ^a	0.46 \pm 0.21 ^{a,m}	
		MON15985	58	0.82 \pm 0.05 ^c	0.86 ^b	0.81 ^b	0.65 ^b	0.39 ^{b,m}	11.1 \pm 0.6 ^{c,m}	14.4 \pm 0.9 ^{b,m}	1.29 \pm 0.51 ^{b,m}	

Numbers within columns and species not followed by a common letter are significantly different ($P < 0.05$; PROC GENMOD [protected lsmeans] or PROC GLM, [Student-Newman-Keuls]; SAS Institute 1998). n, Number of insects tested, including approximately one half which were transferred to artificial diet after 48 h. Non-italicized superscripted letters indicate significant effects of assay method for indicated cultivar (m) or an interaction (i) between cultivars and assay methods.

^a Relative feeding (0–5 scale, \pm SE) 48 h after plant parts were infested.

^b Pupation delay = delay in days relative to DPL50. No bollworm larvae survived to the pupal stage on MON15985.

^c Entire white flowers, including bracts and all floral parts.

^d Fully expanded leaves 4–6 nodes below the uppermost node.

MON15985 was only different from DPL50 on day 3. In all assays, the amount of feeding on the non-Bt cultivar (DPL50) was higher than on Bt cultivars. Both fall armyworms and beet armyworms fed less on tissue from MON15985 than on tissue from DPL50B.

The dual-toxin cultivar also had greater sublethal effects than did the single-toxin cultivar (Table 2). After 5 and 7 d, there was a reduction in the size of beet and fall armyworm larvae that were fed MON15985 versus DPL50 and DPL50B, and a corresponding delay in pupation was observed. Bollworm size was reduced by feeding on either of the Bt cultivars. No bollworms survived when fed MON15985, so the effect of this variety on time to pupation could not be determined. However, DPL50B caused a significant delay in bollworm pupation (8.6 d) relative to DPL50.

Generally, cultivar effects on survival and larval development were greater when larvae were fed plant materials continuously than when transferred to diet after 48 h. Across all comparisons, the assay method had a significant impact on survival to the pupal stage ($\chi^2 = 14.5$, $df = 1$, $P < 0.01$) and on other variables measured in our experiments. Fewer larvae survived to the pupal stage when continuously fed plant material (50%) than when transferred to diet (66%). Similarly, 5 d after originally infested on plant tissue, larvae that had been transferred to diet were larger (17.9 ± 0.3 mm) than those kept on tissue (14.6 ± 0.3 mm) ($F = 70.6$; $df = 1$, 378; $P < 0.01$).

Interactions between cultivar effects and assay method were detected for survival (e.g., survival to pupal stage across all comparisons: $\chi^2 = 7.8$, $df = 2$, $P = 0.03$) (Table 2). These interactions were related to the recovery of insects, after exposure to toxic plant tissue, when they were transferred to artificial diet. For plant tissue that was relatively nontoxic, transferring larvae to diet had little effect on survival. For example, beet armyworm survival to pupation was only 3% when insects were continuously fed plant material from MON15985. Survival in the MON15985 treatment was higher (75%) when beet armyworms were transferred to diet after 48 h ($\chi^2 = 16.0$, $df = 1$, $P < 0.01$), whereas the assay method did not affect beet armyworm survival for DPL50 and DPL50B cotton ($\chi^2 = 0.10$ and 0.01, $df = 1$, $P = 0.72$ and 0.91, respectively). Similar treatment interactions on larval length and pupation delay were detected for beet and fall armyworm. For example, fall armyworms continuously fed flowers of MON15985 and DPL50B were 13.0 ± 0.6 and 18.0 ± 0.6 mm long after 5 d, respectively. Larvae that had been transferred to diet after 48 h were larger (15.4 ± 0.6 and 19.9 ± 0.7 mm long on MON15985 and DPL50B, respectively). In contrast, 5-d larval lengths were similar for fall armyworms continuously fed DPL50 and for those transferred to diet (19.7 ± 0.7 and 18.8 ± 0.6 mm, respectively).

Fresh Tissue Assays (Third-Instar Bollworm). It appears that if bollworm larvae survive to the third instar by feeding on relatively nontoxic hosts or plant parts, they may then be able to survive and damage young bolls from dual-toxin Bt cultivars (Table 3). For any cultivar, no mortality was observed for larvae

Table 3. Proportion of third instar bollworms that survived and penetrated 4-d-old bolls bolls when fed cotton containing zero (DPL50), one (NuCOTN 33B, Cry1Ac), or two Bt proteins (MON15985, Cry1Ac and Cry2Ab)

Cultivars	Surviving larvae, Boll penetration			n
	2 d	4 d	6 d	
DPL50	1.00a, 1.00a	1.00a, 1.00a	0.93a, 1.00a	15
NuCOTN 33B	1.00a, 0.82ab	0.74b, 0.86a	0.63b, 0.86a	35
MON15985	1.00a, 0.68b	0.72b, 0.80a	0.64ab, 0.80a	25
χ^2 (df = 2) ^a	0.00, 8.79	8.20, 5.17	6.26, 5.17	
P	1.00, 0.01	0.02, 0.08	0.04, 0.08	

Data were analyzed using PROC Freq (SAS Institute 1998). Numbers within columns not followed by a common lower letter are significantly different (Fisher's exact test [two-tailed]; $P < 0.05$; Steel and Torrie 1980). n, Number of insects tested.

^a Likelihood ratio chi-square (PROC Freq, SAS Institute 1998).

feeding on bolls for 2 d, and the ability to successfully penetrate the external boll wall was high ($\geq 68.0\%$). While there was no difference in boll penetration between single and dual-toxin Bt cultivars, boll penetration was significantly reduced for the dual-toxin cultivar compared with the non-Bt cultivar. There were no significant differences in boll penetration between cultivars at 4 d, but larvae on both Bt cultivars had lower survival rates than the non-Bt cultivar. After 6 d, larval survival on Bt cultivars was similar and remained somewhat lower than survival on non-Bt cotton, but there was no difference in boll penetration among cultivars ($P > 0.05$).

Assays with Lyophilized Leaf Tissue (Neonate Bollworm and Tobacco Budworm). For all doses of plant material incorporated into the diet, average mortality of tobacco budworm and bollworm never exceeded 26% at 7 d. Thus, the doses used in this experiment were largely sublethal for this time frame. Across all cultivars and for both species, mortality only increased by $\approx 5\%$ between 3 and 7 d (bollworm: $\chi^2 = 4.0$, df = 1, $P < 0.05$; budworm: $\chi^2 = 4.7$, df = 1, $P < 0.05$).

Despite the fact that the doses chosen were mostly sublethal, cultivar and dose significantly influenced the mortality of bollworm at 3 d ($\chi^2 = 34.1$, df = 2, $P < 0.01$ and $\chi^2 = 30.5$, df = 3, $P < 0.01$, respectively) and 7 d ($\chi^2 = 44.1$, df = 2, $P < 0.01$ and $\chi^2 = 26.4$, df = 3, $P < 0.01$, respectively). Bollworm mortality for MON15985 was about 9–13% higher than for the other cultivars on days 3 and 7. No significant difference in mortality was detected between DPL50 and DPL50B (Table 4). Lower dosages (1.124 and 11.24 $\mu\text{g}/\text{ml}$ diet) tended to cause less mortality of bollworms at both three and 7 d. An interaction between cultivar and dose was detected 3 d after bollworm larvae were infested on treated diet ($\chi^2 = 16.8$, df = 6, $P = 0.02$), but not at 7 d ($\chi^2 = 11.7$, df = 6, $P > 0.07$). At 3 d, survival of bollworms fed 1.124, 11.24, 112.4, and 1124 μg of DPL50 plant tissue/ml of diet was 93.1, 91.4, 83.8, and 80.8%, respectively. For DPL50B, survival at these same dosages was 93.6, 91.4, 81.7, and 85.3%, respectively. So higher dosages tended to cause more mortality for these varieties, even for the non-Bt cultivar. Bollworm survival on MON15985 was 84.5, 73.2, 75.9, and 80.6% as dosages were increased. Thus, a clear dose-response pattern was not evident for the MON15985 cultivar, with greater mortality actually occurring at intermediate dosages.

Tobacco budworm survival was not significantly influenced by cultivar on either day 3 or 7 ($\chi^2 = 5.3$, df = 2, $P > 0.07$ and $\chi^2 = 2.81$, df = 2, $P > 0.24$, respectively), and no interactions were found between treatment and dose for either day ($\chi^2 = 6.9$, df = 6, $P > 0.33$ and $\chi^2 = 5.7$, df = 6, $P > 0.46$, respectively). Despite the lack of cultivar effects on survival, there was a significant dose-response on day 7 ($\chi^2 = 9.8$, df = 3, $P = 0.03$), with $\approx 8\%$ more mortality occurring at the highest dose compared with the lowest dose (Table 4).

Sublethal effects on larval development (i.e., length) were greatly influenced by cultivar and dose for both species. Cultivar effects on bollworm ($F = 197$; df = 2, 563; $P < 0.01$) and budworm ($F = 100$; df = 2, 563; $P < 0.01$) were similar in that larvae fed MON15985 were typically smaller than larvae fed DPL50B, and larvae fed non-Bt cotton were larger than those fed either of the Bt cultivars (Table 5). The dose-response for bollworm and budworm also were similar (bollworm: $F = 62.6$; df = 3, 563; $P < 0.01$, and budworm: $F = 76.0$; df = 3, 563; $P < 0.01$) in that larvae fed higher doses were generally smaller than those fed lower doses. However, there was an obvious interaction between cultivar and dose for both species (bollworm: $F = 6.81$; df = 6, 563; $P < 0.01$, and budworm:

Table 4. Proportion of neonate bollworm and tobacco budworm that survived after being fed lyophilized leaf tissue from cotton containing zero (DPL50), one (DPL50B, Cry1Ac), or two Bt proteins (MON15985, Cry1Ac and Cry2Ab)

Analysis variable	Bollworm survival		Budworm survival	
	3 d ^a	7 d	3 d	7 d
Cultivar DPL50	0.87a	0.85a	0.82a	0.78a
DPL50B	0.88a	0.81a	0.78a	0.75a
MON15985	0.79b	0.72b	0.82a	0.72a
Dose ^b				
1.124	0.90a	0.85a	0.83a	0.81a
11.24	0.85b	0.81ab	0.80a	0.78ab
112.4	0.80c	0.75c	0.81a	0.78ab
1124	0.82bc	0.76c	0.79a	0.73b

Leaf tissue was incorporated into diet at four different doses.

Means without columns not followed by a common letter are significantly different ($P < 0.05$, PROC Genmod, protected lsmeans, SAS Institute 1998).

^a Indicates a significant interaction, for bollworm, between cultivar and dose on day 3 (see text).

^b μg of plant tissue per ml of artificial diet.

Table 5. Mean length (mm \pm SE) of bollworm and tobacco budworm larvae after 7 d when neonates were put on diet containing lyophilized leaf tissue at four different doses

Dose ^a	Bollworm length			Budworm length		
	DPL50	DPL50B	MON15985	DPL50	DPL50B	MON15985
1.124	15.7 \pm 0.3Aa	14.9 \pm 0.3Ba	11.0 \pm 0.7Ca	13.2 \pm 0.3Aa	13.0 \pm 0.4Aa	13.6 \pm 0.6Aa
11.24	15.6 \pm 0.4Aa	13.6 \pm 0.3Bb	11.2 \pm 0.6Ca	13.5 \pm 0.4Aa	13.0 \pm 0.4ABa	12.4 \pm 0.5Ba
112.4	14.3 \pm 0.4Ab	13.1 \pm 0.5Bb	8.1 \pm 0.5Cb	13.3 \pm 0.4Aa	12.4 \pm 0.3Ba	8.7 \pm 0.4Cb
1124	13.9 \pm 0.5Ab	9.5 \pm 0.3Bc	5.1 \pm 0.3Cc	13.2 \pm 0.4Aa	10.2 \pm 0.3Bb	4.4 \pm 0.3Cc

Leaves were from cotton containing zero (DPL50), one (DPL50B, Cry1Ac), or two Bt proteins (MON15985, Cry1Ac and Cry2Ab).

Means within a row and species not followed by a common uppercase letter are significantly different, and means within columns not followed by a common lower letter are significantly different ($P < 0.05$, PROC GLM, Student-Newman-Keuls, SAS Institute 1998).

^a μ g of plant tissue per ml of artificial diet.

$F = 34.5$; $df = 6, 563$; $P < 0.01$). Compared with DPL50B and MON15985, increasing the amount of DPL50 tissue incorporated into diet had a relatively small impact on larval growth. The dose-response for MON15985 was particularly evident for both species compared with the other varieties.

Discussion

It is uncertain how much additional protection from insects the insertion of this second Bt toxin-producing gene will give to cotton until it is introduced and used on a large scale. However, our data suggest that dual-toxin Bt cottons will provide substantially better control of lepidopteran pests compared with the existing, single-toxin Bt cultivars. In field studies, Stewart et al. (2000; unpublished data) have shown that the dual-toxin Bt cultivar (MON15985) provided a significant improvement in control of bollworm, soybean looper, and fall armyworm populations relative to the single-toxin cultivar (DPL50B). The superiority of cultivars expressing both Cry1Ac and Cry2Ab should be most apparent for soybean loopers, beet armyworms, and fall armyworms because these species are less affected than bollworm by the Cry1Ac single-toxin Bt cultivars that are presently available. The available, single-toxin Bt cottons sometimes require applications of foliar insecticides to control soybean looper, beet armyworm, fall armyworm, and bollworm. Based on these results and that of Stewart et al. (2000; unpublished data), the dual-toxin cultivars (expressing Cry1Ac and Cry2Ab) may not require supplemental insecticide applications for these pests.

Relative to the single-toxin Bt cultivar, the dual-toxin cultivar had similar and negative impacts on growth of both bollworm and tobacco budworm (Tables 4 and 5). This will have clear implications in managing resistance to Bt cotton for these pests (see Tabashnik 1994, Sachs et al. 1996, Gould 1998). Interestingly, a relatively small but significant dose-response was observed for bollworm when fed tissue from non-Bt cotton. Naturally occurring toxins in cotton leaves, such as gossypol, may have caused this effect (Lukefahr et al. 1975).

The interactions we detected between cultivar effects and assay method, for fresh tissue assays (Table 2), could indicate that one of the assay methods was not as reliable in separating treatment effects. These

interactions resulted from the recovery of larvae after they were transferred from Bt-cotton tissue to diet (particularly MON15985), whereas recovery did not occur when insects were continuously fed plant material. Despite the interactions, similar conclusions concerning cultivar effects on larval survival and development would generally be made based on the results of either assay method. However, cultivar effects were more dramatic when larvae were kept on plant tissue throughout development. Thus, maintaining insects on plant material, rather than transferring to diet, appears the best approach for increasing the sensitivity of fresh-tissue assays.

Because we did not test a cultivar expressing Cry2Ab alone, we cannot conclude that Cry2Ab had greater activity on armyworms than the Cry1Ac toxin in DPL50B. Also, the amount of Bt toxins, Cry1Ac and Cry2Ab, expressed in the plant tissue was not determined. Greenplate et al. (2000) reported similar levels of Cry1Ac in near-isogenic lines of cotton expressing either Cry1Ac alone or Cry1Ac and Cry2Ab (referred to as "CryX" in his article). However, the level of Cry2Ab expression in plant tissue was ≈ 10 times that of Cry1Ac. The potency of Cry1Ac versus Cry2Ab, at equivalent doses, varies for different lepidopteran species (Sims 1997). Furthermore, the dose received by an insect was self-regulated in our assays. Nonpreference for the various toxic plant tissues may influence the dose ingested by individuals. Insects fed higher doses of Bt toxins may actually feed less as a result of gut paralysis (Sikorowski and Lawrence 1997), and thus, actually consume a smaller dose of plant tissue. Reduced feeding on Bt tissue was obvious in assays using fresh plant parts. So for our assays, these factors make it difficult to compare the sensitivity of different species to the various Bt toxins (or compare among 'doses' within a given assay) because the ingested dose was not known. However, assays with fresh tissue or lyophilized tissue were well suited for comparing the relative toxicity of plant tissues.

Reductions in larval length and pupation delays observed in fresh-tissue and lyophilized-tissue assays were most diagnostic of increased toxicity in the dual-toxin cultivars. Besides indicating the direct effects of the Bt toxins, these sublethal variables may also reflect the indirect impact of reduced feeding on toxic plant tissue. Assays using lyophilized plant tissue incorporated into diet have some advantages because plant

tissues could be stored until needed. This allows for comparison among plant tissues collected at different times (e.g., early versus late season). Also, there is no need to periodically give fresh plant material to the insects. An additional advantage is that dosages can be adjusted so that excessive mortality caused by toxic plant tissue does not occur, and hence neonates can be used in assays. With fresh plant tissue, particularly with neonates, excessive mortality may make separating treatment effects (i.e., Bt cultivars) statistically difficult.

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